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# Imaging Vascular Endothelial Activation: An Approach Using Radiolabeled Monoclonal Antibodies Against the Endothelial Cell Adhesion Molecule E-Selectin

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E-selectin is an endothelial cell-specific adhesion molecule for leukocytes expressed on the luminal surface of vascular endothelium during inflammatory responses. Because E-selectin expression is dependent upon ongoing stimulation by cytokines, this molecule offers a potentially useful target for imaging tissues in disease states involving cytokine-mediated endothelial cell activation. **Method:** To assess the imaging potential of an anti-E-selectin monoclonal antibody (Mab) 1.2B6, the accumulation of intravenously injected  $^{111}\text{In}$ -labeled Mab 1.2B6 was compared to that of  $^{111}\text{In}$ -control antibody in a model of arthritis in the pig. Injection of phytohaemagglutinin (PHA) into a knee led to E-selectin expression on vessels in the synovium and draining deep inguinal lymph nodes, as demonstrated by immunohistology. No E-selectin expression was seen in the control knee injected with buffer alone. Animals were given  $^{111}\text{In}$ -Mab 1.2B6 or  $^{111}\text{In}$ -control antibody intravenously 3 hr after the intra-articular injection of PHA. Radiolabeled antibody uptake was measured by direct counting of tissues 25 hr postmortem. **Results:** The accumulation of radiolabeled control IgG in synovium and draining deep inguinal lymph nodes of PHA-injected knees was significantly higher than accumulation in tissues injected with buffer alone; however, the comparable ratios in animals receiving radiolabeled Mab 1.2B6 were significantly greater. Scintigraphy performed 24 hr after  $^{111}\text{In}$ -Mab 1.2B6 injection showed obvious localization of activity in the inflamed knee in each of three animals. **Conclusion:** Radiolabeled anti-E-selectin Mab can be used to image localized inflammatory tissues. This approach may be useful for investigating activated endothelium in human disease.

**Key Words:** inflammation; vascular endothelial activation; endothelial cell adhesion; E-selectin

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Over the last few years there has been a great increase in our understanding of the active role played by endothelium in the orchestration of inflammatory responses. In particular, endothelial cells (EC) respond to inflammatory stimuli by de novo expression of a number of surface antigens and soluble mediators (1). Because of the immediate accessibility of endothelium to the blood, this new understanding of the nature of endothelial surface activation antigen expression in inflammation offers attractive possibilities for imaging. Thus, radiolabeled monoclonal antibodies (Mab) against EC activation antigens might be useful for the noninvasive evaluation of endothelial activation in diverse clinical situations. Such precise molecular targeting would represent an advance over techniques that nonspecifically image inflammation such as  $^{67}\text{Ga}$ -citrate or polyclonal IgG.

E-selectin (endothelial-leukocyte adhesion molecule-1, ELAM-1) is an EC activation antigen which acts as an adhesion molecule for the recruitment of circulating leukocytes into the tissues during inflammatory responses (2,3). It is a single-chain glycoprotein that is not expressed by resting EC but which is induced in vitro following stimulation of EC by proinflammatory mediators such as interleukin-1, tumor necrosis factor or bacterial lipopolysaccharide (4-6). As E-selectin expression is limited to activated endothelium (7,8), this molecule could be an ideal imaging target for diagnostic radioimmunoscintigraphy.

We have recently demonstrated (9) that intravenously injected  $^{111}\text{In}$ -labeled anti-E-selectin Mab is taken up into inflammatory sites in pig skin stimulated with interleukin-1, tumor necrosis factor or phytohaemagglutinin (PHA) (10), correlating with leukocyte accumulation and endothelial expression of E-selectin observed immunohistologically (9,11). This uptake of anti-E-selectin was specific as there was no increase in uptake of  $^{99\text{m}}\text{Tc}$ -labeled control IgG into the same inflammatory sites. In this study, we utilized this technique to image a localized inflammatory response us-

ing a model of arthritis induced by intra-articular injection of PHA (12).

## METHODS

### Monoclonal Antibodies

Mab 1.2B6 is a mouse IgG<sub>1</sub> Mab against human E-selectin (13). This antibody also recognizes porcine E-selectin, as shown by specific reactivity with COS cells transfected with porcine E-selectin cDNA (Y. Tsang, unpublished data). Mab 1.2B6 was purified from tissue culture supernatant by Protein A affinity chromatography (14). The control reagent MOPC 21 is a mouse IgG<sub>1</sub> myeloma protein (15) of undefined specificity and was the kind gift of Dr. Martyn Robinson (Celltech Ltd., Slough, England).

### Antibody Labeling with <sup>111</sup>In

Antibodies were labeled with <sup>111</sup>In using the method described by Hnatowich et al. (16). The antibodies were first coupled with the bicyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) (D 6148, Sigma Chemical Co. Ltd., Dorset, U.K.). A 5-mg aliquot of antibody in 1 ml 0.1 M bicarbonate buffer, pH 8.0, was added to dry DTPA to give an antibody-to-DTPA molar ratio of approximately 1:10. After an incubation period of 30 min at room temperature, the coupled antibody was separated from free DTPA by gel filtration on a Sephadex G50 (Pharmacia LKB Biotechnology, Uppsala, Sweden) column in a 20-ml sterile syringe. The column was eluted using 0.1 M sodium acetate, pH 6.0. Protein concentration was measured using a spectrophotometer (Ultrospec II, Pharmacia LKB Biotechnology, Uppsala, Sweden) and the peak aliquots were pooled and filtered through a 2- $\mu$ m microfilter (Ministart, Sartorius GmbH, Gottingen, Germany).

The DTPA-coupled antibodies (~1 mg/ml) were stored in 200- $\mu$ l aliquots at 4°C ready for subsequent labeling. On the morning of each study, 25 MBq of chelation grade <sup>111</sup>In (indium chloride in 0.04 M HCl, carrier free; INS 1, Amersham International plc, Amersham, U.K.) was brought to pH 6 by the addition of 3.8% sodium citrate, pH 7.4. This was then added to an aliquot of the DTPA-coupled antibody and incubated for 30 min at room temperature. Radiolabeled antibody was separated from free <sup>111</sup>In by gel filtration on a Sephadex G50 column eluted with PBS. The conjugation and labeling protocol yielded antibodies with 0.8–1.2 DTPA molecules per antibody molecule and a specific radioactivity of 100–150 MBq/mg. The efficiency of protein binding of <sup>111</sup>In was >90%, as judged by thin-layer chromatography. No change in protein-bound radioactivity was observed when thin-layer chromatography was repeated after 24 hr, demonstrating a high degree of stability of the conjugates. The binding constant of <sup>111</sup>In-labeled Mab 1.2B6 was 4.8 nM when assessed by Scatchard analysis using tumor necrosis factor-activated human umbilical vein endothelial cells.

### Animals

Six healthy young large white pigs weighing 15–25 kg were obtained from a commercial supplier. Animals were housed individually under standard husbandry conditions and studied according to a protocol approved under the United Kingdom Animals (Scientific Procedures) Act 1986.

### Model of PHA-Induced Arthritis

Animals were anesthetised for the intra-articular injections and subsequent imaging studies. Anesthesia was induced using halothane by inhalation resulting in rapid induction of sedation with minimal stress. Anesthesia was maintained by repeated intrave-

nous boluses of propofol (1 mg/kg; Diprivan; ICI Pharmaceuticals, Macclesfield, U.K.), given every 15–20 min. The animals did not require intubation or other external support.

Based on previous work looking at lymphocyte traffic into localized inflammatory lesions (10), 400  $\mu$ g of PHA (L-8754, Sigma Chemical Co. Ltd., Dorset, U.K.) in 1 ml RPMI 1640 was injected into the test knee and 1 ml RPMI 1640 was injected into the control side. Three hours after the intra-articular injections, 100  $\mu$ g of either <sup>111</sup>In-labeled anti-E-selectin Mab 1.2B6 (three animals) or <sup>111</sup>In-labeled control antibody (three animals) were given as an intravenous bolus. Animals were screened in the supine position under a gamma camera (IGE Starport, GE Medical Systems, Milwaukee, WI) for 90 min after antibody injection. Initially, dynamic imaging of the lower abdomen and hind limbs was performed using a 1-min frame rate for 60 min. Following this, a 15-min static image was obtained. A final 30 min static image of the lower abdomen and hind limbs was obtained 24 hr after the intra-articular injections.

At the end of each study, animals were killed by overdose of anesthetic. The deep inguinal, superficial inguinal and popliteal lymph nodes and the synovial lining of the knee joints were excised immediately postmortem and were placed in previously weighed containers. The radioactivity was then counted in a well counter (NM108; J + P Engineering, Reading, England) and background counts subtracted. Accumulation of radioactivity was expressed as a “localization ratio” (LR). For the lymph nodes, the LR was the CPM/g for each group (deep inguinal, superficial inguinal or popliteal) from the PHA-injected side divided by the CPM/g of the equivalent group from the control side. Since the extent of inflammation in the knee synovium was difficult to define with the naked eye, accumulated radioactivity was counted in the total excised synovium together with a margin of connective tissue. The LR of synovium was then calculated as the total CPM in the counted tissue from the PHA-injected knee divided by the total CPM in the counted tissue from the control knee. The ratio of CPM/g for small synovial biopsies from each knee was calculated for animals 2, 3, 5 and 6 and the LR values did not differ significantly from the values given for the LR calculated from the counts in the total excised tissue (data not shown).

### Immunohistochemical Studies

After measuring the radioactivity in the tissues as described above, multiple samples were embedded in OCT compound (Miles Laboratories Inc., Elkhart, IN), snap frozen and stored at –70°C for subsequent staining. Cryostat sections (7–10  $\mu$ m) were mounted on poly-L-lysine-coated slides, air-dried for 2–3 hr and fixed in 50% methanol/50% acetone for 5 min. Staining was done using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method and antibody-binding was visualized using Fast Red substrate by a modification (11) of a previously described method (17). The degree of inflammation and the intensity of E-selectin expression was scored blindly by an experienced observer, using a semiquantitative scale of 0 (no detectable staining), + (weak staining of occasional vessels) and ++ (widespread moderate–strongly stained vessels).

### Statistics

The degree of localization of labeled antibodies in inflamed synovia and regional lymph nodes was assumed to be normally distributed between pigs. Uptake of <sup>111</sup>In-labeled Mab 1.2B6 was therefore compared with uptake of <sup>111</sup>In-labeled control antibody using an unpaired Student's t-test.

**TABLE 1**  
The Uptake of  $^{111}\text{In}$ -Labeled Mab 1.2B6 or Control Antibody into Synovium and Deep Inguinal Lymph Nodes and Immunohistochemical Staining for E-Selectin at These Sites

Animal no.	$^{111}\text{In}$ -labeled antibody	Localization ratio*		E-selectin expression†			
		Synovium	Deep inguinal lymph nodes	Knee		Deep inguinal lymph nodes	
				PHA	Control	PHA	Control
1	Mab 1.2B6	6.8	10.4	++	-	++	+
2	Mab 1.2B6	16.9	5.2	++	-	++	-
3	Mab 1.2B6	16.1	10.0	++	-	++	-
		$13.3 \pm 5.6$ ‡§	$8.5 \pm 2.9$ ‡§				
4	Control IgG	1.3	0.9	++	-	++	-
5	Control IgG	3.0	1.4	++	-	++	+
6	Control IgG	3.7	1.2	++	-	++	-
		$2.7 \pm 1.2$	$1.2 \pm 0.3$				

\*LR values were calculated as described in the Methods section.

†The degree of E-selectin expression in frozen sections was scored blindly by an experienced observer.

‡Mean  $\pm$  s.d.

§ $p < 0.05$  (Student's t-test) compared with animals that received  $^{111}\text{In}$ -labeled control IgG.

## RESULTS

While the intra-articular injection of PHA produced no external signs of inflammation, the synovial lining of PHA-injected joints appeared inflamed when the cavity was opened postmortem and there was often a small synovial effusion noted. In addition, the deep inguinal lymph nodes draining the inflamed knee appeared reactive, especially in the posterior portion of the chain. No macroscopic changes were detected in the popliteal and superficial inguinal lymph nodes on the side of either the PHA-injected or the control knees.

Immunohistochemical staining of tissue sections was performed to confirm activation of endothelium and expression of E-selectin in all pigs used in the study (Table 1). The synovial lining (Fig. 1A) of the joints injected with buffer alone showed no evidence of anti-E-selectin Mab 1.2B6 staining or of an inflammatory cell infiltrate. In contrast, Mab 1.2B6 clearly stained the endothelium of venules in the synovium of the PHA-injected joint and these positive vessels were surrounded by a marked infiltration of inflammatory cells (Fig. 1B). In addition, there was a marked increase in Mab 1.2B6 staining of endothelium in the deep inguinal lymph nodes draining the PHA-injected joints. The popliteal and superficial inguinal lymph nodes showed no expression of E-selectin, on the side of either PHA-injected or control knees.

A modest increase in uptake of radiolabeled antibody in the inflamed knee relative to the control knee was evident during the 1-hr dynamic study and in the early 15-min static image using either Mab 1.2B6 or control IgG (data not shown). The delayed static images obtained at 24 hr showed that the image intensity in the PHA-injected joint

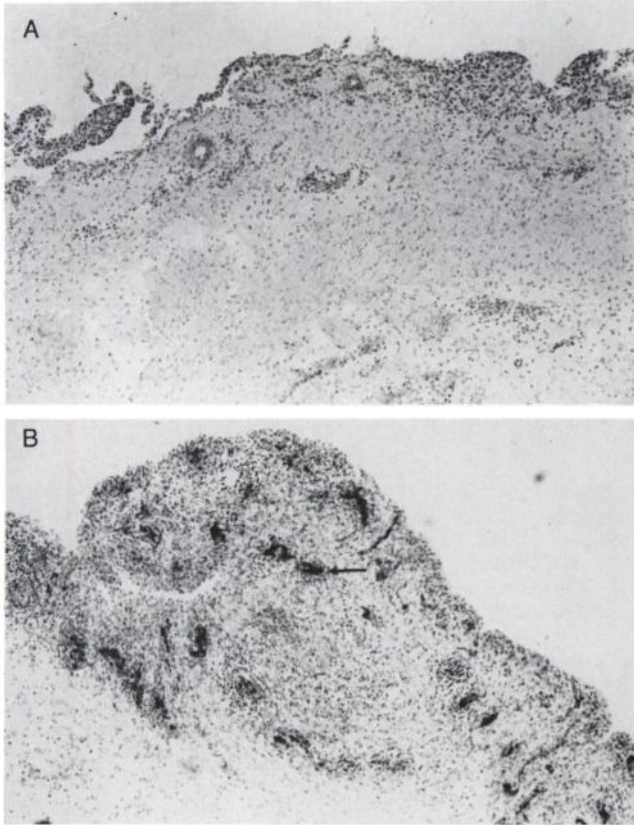
had continued to increase and this uptake was markedly greater for the anti-E-selectin Mab than for the control antibody (Fig. 2). In the case of radiolabeled Mab 1.2B6, the joint localization was so intense as to mask the normal appearance of the joint space on the gamma camera image.

In addition to the greater localization in the joint itself, there was also evidence of greater clearance of the Mab 1.2B6 background radioactivity at 24 hr when compared with radiolabeled control antibody. This was particularly marked in one pig (animal 1, Table 1), in which there was clear imaging of the ipsilateral deep inguinal lymph nodes by 24 hr (Fig. 2B). Figure 2B also shows imaging of the skin, visible as a distinct edge to the body outline.

In order to validate the greater localization of radioactivity in animals that had received Mab 1.2B6, tissues were excised and radioactivity counted postmortem. Whereas the LR for radiolabeled control IgG in synovium and draining iliac lymph nodes were  $2.7 \pm 1.2$  and  $1.2 \pm 0.3$  (mean  $\pm$  s.d.) respectively, those for radiolabeled Mab 1.2B6 were significantly greater at  $13.3 \pm 5.6$  ( $p < 0.05$ ) and  $8.5 \pm 2.9$  ( $p < 0.05$ ) (Table 1). There were no differences between pigs that received radiolabeled control antibody and those that received radiolabeled Mab 1.2B6 in LR of popliteal and superficial inguinal lymph nodes.

## DISCUSSION

A number of different approaches to the imaging of inflammation have been developed in recent years. Broadly speaking, these may be considered in three groups: (1) those that rely on the nonspecific accumulation at sites of inflammation of radionuclides administered either as salts such as  $^{67}\text{Ga}$ -citrate (18) or coupled to proteins such as



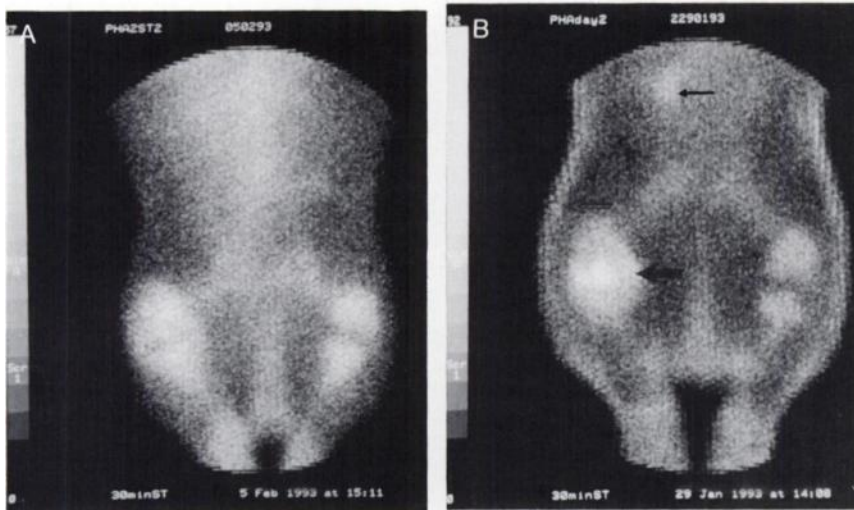
**FIGURE 1.** Immunohistochemical staining of synovium from both test and control knee joints. Tissues were snap frozen and sections were fixed in acetone/methanol before staining for reactivity with anti-E-selectin Mab 1.2B6. (A) There was no E-selectin staining in the synovium from the control knee injected with buffer alone. (B) There was marked endothelial staining (←) of venules in the synovium of the PHA-injected knees and these vessels were surrounded by intense inflammatory cell infiltrates ( $\times 460$ ).

albumin (19) or nonspecific IgG (20); (2) those that detect the uptake of neutrophils, labeled either in vitro with lipophilic chelates (21) or in vivo by anti-granulocyte anti-

bodies (22); and (3) those that target specific ligands on endothelial cells and/or localizing migratory cells using radiolabeled antibodies or peptides. While this third approach has previously been employed using antibodies against IIb/IIIa on platelets (23), P-selectin on platelets and endothelial cells (24), and ICAM-1 on leukocytes and endothelial cells (25), endothelial-specific activation antigens have not been used as targets for imaging purposes. In this study we have demonstrated that it is possible to image inflammation by targeting E-selectin induced on vascular endothelium by the inflammatory process.

The imaging potential of radiolabeled anti-E-selectin Mab 1.2B6 was tested in a model of PHA-induced arthritis, based on previous work demonstrating (1) the accumulation of radiolabeled lymphocytes in synovium following intra-articular injection of PHA (10); (2) the specific localization of anti-E-selectin Mab 1.2B6 to PHA-injected skin sites (9); and (3) immunohistological evidence of E-selectin expression in PHA-injected skin sites (11). Whereas immunohistochemical staining of E-selectin with Mab 1.2B6 was undetectable on the synovial vessels of unstimulated joints, there was clear E-selectin expression on the endothelium of postcapillary venules, together with a marked perivascular leukocytic infiltration, in the synovia of all joints injected with PHA. At present, it is not clear whether induction of E-selectin expression on synovial endothelium in this model is a direct endothelial cell response to PHA or is secondary to the release of cytokines such as interleukin-1 or tumor necrosis factor. In skin, there is evidence that the PHA-induced response is at least partially independent of these two cytokines (10).

We have shown by intra-articular injection of India ink that the deep inguinal rather than the popliteal or superficial femoral lymph nodes drain lymph from the knee (data not shown). Consistent with this observation, we found that vessels in the deep inguinal lymph nodes draining PHA-injected knees expressed E-selectin, whereas no E-selectin induction was detected by immunohistochemis-



**FIGURE 2.** Thirty-minute static gamma camera images showing anterior views of the lower abdomen and hind limbs acquired 24 hr after the intra-articular injection of PHA (400  $\mu$ g) into the right knee and buffer into the left knee. The images show the pattern of uptake of (A)  $^{111}\text{In}$ -labeled control antibody and (B)  $^{111}\text{In}$ -labeled anti-E-selectin Mab 1.2B6 given intravenously 3 hr after the intra-articular injections. There is increased uptake of both antibodies into the PHA-injected joint though this is considerably more diffuse and less intense for the control antibody than for anti-E-selectin Mab 1.2B6 (←). In addition, Mab 1.2B6 is taken up into the deep inguinal lymph nodes draining the inflamed knee (↔) and appears also to be taken up by the skin, visible as a margin to the body outline (↔).



try in vessels of ipsilateral popliteal or superficial inguinal lymph nodes. This vascular endothelial activation of vessels in the regional lymph nodes was probably due to the lymphatic passage either of PHA itself or of cytokines generated by the articular inflammatory response. It was unlikely to be due to vascular dispersal of these factors as the contralateral deep inguinal lymph nodes showed minimal expression of E-selectin.

Specific uptake of anti-E-selectin compared to that of control immunoglobulin in PHA-injected joints was clearly observable with the naked eye in gamma camera images taken 24 hr after injection of radiolabeled antibody and was validated by counting accumulated radioactivity in samples of synovium excised postmortem. Based upon in vitro work, it is probable that the accumulation of radiolabeled anti-E-selectin depends not only upon the degree of expression of E-selectin by vessels in inflamed tissues but also on progressive internalization of antibody-antigen complexes following binding of the Mab to endothelium (26). Insofar as the control immunoglobulin might be expected to reflect the behavior of radiolabeled human immunoglobulin in inflammatory disease, the marked difference between localization of anti-E-selectin Mab 1.2B6 and the control immunoglobulin predicts a superiority of such an antibody over polyclonal human immunoglobulin for clinical imaging.

Apart from the more intense image of the inflamed knee obtained with the radiolabeled anti-E-selectin, there was also a greater clearing of the background radioactivity compared with that observed with control immunoglobulin. This was particularly marked in one animal (animal 1), in which the decreasing background in parallel with the increasing localization of signal rendered possible the clear imaging of the deep inguinal lymph nodes at 24 hr postinjection. It is possible that in this instance the clearing of Mab 1.2B6 was due to specific uptake in the skin, which we have shown elsewhere to express variable amounts of E-selectin in the absence of experimental stimulation (9,11). Nevertheless, this ability of the antibody to clear from the circulation may be a distinct advantage for the clinical imaging of a localized lesion in internal organs.

Because E-selectin functions as an adhesion molecule for leukocytes, in some respects imaging inflammation with radiolabeled anti-E-selectin can be compared with imaging with radiolabeled leukocytes. Although this latter technique has an established role in the clinical investigation of inflammatory disorders, the preparation of radiolabeled leukocytes is time-consuming and requires experience. For this reason, labeled white cell scans are not generally available outside specialist centers. Since imaging with radiolabeled antibody E-selectin is technically more straightforward, it will now be important to evaluate the potential of this technique in the clinical setting in a wide variety of cardiovascular, rheumatic and neoplastic disorders involving cytokine-mediated endothelial activation.

## ACKNOWLEDGMENTS

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## EDITORIAL

# Imaging Vascular Endothelial Activation

Keelan and colleagues exploit some of the recently acquired knowledge in molecular biology, particularly the fundamentally important mechanisms of leukocyte-endothelial adhesion in the inflammatory reaction (1). A brief summary of this subject (2-5) follows for the stout-hearted (others, please skip the next paragraph).

Of all leukocyte integrin adhesion molecules, only neutrophils activated in inflammatory lesions by local specific chemotactic cytokines such as interleukin-8 (IL-8) possess significant numbers of the surface glycoprotein complexes designated CD11b/CD18 or Mac-1. These complexes interact with endothelial intercellular adhesion molecule-1 (ICAM-1). Another leukocyte integrin, LFA-1, predominantly in lymphocytes but also in neutrophils, has an affinity for endothelial ICAMs (5). Other adhesion molecules include three selectins: L-selectin or leukocyte endothelial cell adhesion molecule 1 (lymphocyte homing receptor, MEL-14, gp 90, LAM-1 or Leu 8) is expressed on the surface of neutrophils; E-selectin or endothelial leukocyte adhesion molecule (ELAM-1), expressed on the surface of activated endothelial cells (as described by the authors), interacts with L-selectin, leading to cell margination which is followed by adhesion as a result of the interaction between the neutrophil integrin and ICAM-1; and P-selectin, granule membrane protein-140 (GMP-140) or platelet activation-dependent granule to external membrane (PADGEM, CD62) is released from platelets and endothelial cells, promoting further adhesion of neutro-

phils and platelets. Thus, adhesion is a multistep process involving multiple alternative receptor-ligand pairs (5), and all of the players have not been identified as yet.

In this preliminary paper, Keelan et al. describe an intriguing idea for imaging foci of inflammation with a radiolabeled monoclonal antibody (Mab) specifically for E-selectin expressed on the surface of activated endothelial cells. So far, they have provided quantitative data only in the form of localization ratios of inflamed versus contralateral normal extremities in pigs. Information on the absolute concentration of radioactivity in different inflammatory lesions, including abscesses, also would be important. How would these concentrations compare with those obtained with leukocytes labeled in vitro after their re-injection? To assess the value of this agent, we need to know its distribution in the major visceral organs to judge its efficacy in detecting inflammatory lesions in the torso. Some information on the cardiac, hepatic and skin activity, and plasma disappearance is provided in the companion paper (author's reference 9). Immunohistological studies in this earlier publication showed that the only normal tissue expressing E-selectin was the vascularity of the dermis.

This imaging approach unavoidably introduces the well-known disadvantages of murine Mabs such as the likelihood of HAMA formation. The plasma clearance of the large IgG<sub>1</sub> molecules will be slow. Hence, optimal imaging will probably be seen at 24 hr instead of providing definitive images within a few hours. However, Keelan et al. found that the plasma clearance was faster with the specific Mab than with nonspecific IgG, prob-

ably because of better localization in the inflammatory lesion and extraction by the dermal vasculature. Could specific antibody Fab, Fab<sub>1</sub> or F(ab)<sub>2</sub> fragments reduce the time interval for optimal imaging?

Many other questions remain to be answered. Is traumatized endothelium activated by mechanisms similar to those of inflammatory lesions? The pathology literature (summarized in companion paper reference 9) indicates that E-selectin expression occurs in many lesions, including chronic dermatoses, "collagen vascular" diseases, allergies, transplant rejection and even lymphoid malignancies. Will leukocytes attracted and bound to activated endothelial E-selectin receptors compete with the binding of specific Mab molecules?

Despite these unknowns, this new approach looks exciting. Hence, further experimental work and subsequent clinical trials appear very worthwhile.

John G. McAfee

The National Institutes of Health  
Bethesda, Maryland

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